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Multicenter Clinical Evaluation of the Automated ARIES® *Bordetella* Assay

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ABSTRACT

Molecular methods offer superior sensitivity and specificity, and reduce testing turnaround time from days to hours for detection of *Bordetella pertussis* and *Bordetella parapertussis*. In this study, we evaluated the performance of the automated PCR-based ARIES *Bordetella* Assay, which detects both *B. pertussis* and *B. parapertussis* directly from nasopharyngeal swab specimens. Limits of detection were 1,800 colony-forming units/mL (CFU·mL^{-1}) for *B. pertussis* and 213 CFU·mL^{-1} for *B. parapertussis*. The assay detected 16/18 unique *B. pertussis* / *B. parapertussis* strains. Of 71 potential cross-reacting organisms, 5 generated false-positives in 1/6 replicates; none of 6 additional *Bordetella* spp. were erroneously detected. Specimens were stable at 20−25°C for at least 10 h, 4−8°C for 10 days, and at ≤ -70°C for 6 months. Of 1,052 nasopharyngeal specimens from patients with suspected pertussis, 3.0% (n=32) were *B. pertussis*-positive, and 0.2% (n=2) were *B. parapertussis*-positive. After combining these data with ARIES *Bordetella* Assay data from 57 nasopharyngeal samples with previously confirmed *B. pertussis* or *B. parapertussis*, and from 50 contrived *B. parapertussis* samples, ARIES assay respective positive and negative percent agreements with the reference assays were 97.1% and 99.0% for *B. pertussis*, and 100% and 99.7% for *B. parapertussis*. The ARIES *Bordetella* Assay provides accurate detection and distinction of *B. pertussis* and *B. parapertussis* infection within 2 hours.

Introduction

Pertussis, or whooping cough, is a highly contagious respiratory disease caused by the Gram-negative bacteria *Bordetella pertussis* and *Bordetella parapertussis*. In some patients, especially infants and young children, infection with these organisms can lead to potentially life-threatening complications such as pneumonia, weight loss, dehydration, and seizures. Although there is a vaccine to prevent disease caused by *B. pertussis*, an estimated 24.1 million cases and over 160,000 deaths, mainly in infants and children less than 5 years old, are recorded annually worldwide (1). In the US, pertussis prevalence has risen since the 1980s, with cyclical peaks occurring every 3–5 years (2). In 2012, the most recent peak year, the Centers for Disease Control and Prevention (CDC) identified 48,277 cases nationwide, though more cases were likely undiagnosed. In 2016, the most recent year with complete statistics, the CDC confirmed nearly 18,000 cases in the US (3).

Disease signs and symptoms caused by these two pathogens are similar; however, those associated with *B. parapertussis* infection are often milder and disease duration is generally shorter (4–7). Both pathogens cause biphasic symptoms that appear following a 5–10-day incubation period. A 7–10-day prodromal stage, the catarrhal stage resembling coryza, progresses to classic pertussis symptoms, which include paroxysmal coughing followed by high-pitched “whooping” during inspiration of air against narrowed airways, and sometimes post-tussive emesis. In adults, signs and symptoms can persist for several weeks. Early and accurate diagnosis of pertussis is essential for the optimization of therapy and to curb the transmission of these pathogens (5–7).

Distinguishing between these two pathogens is important from a public health perspective, because *B. parapertussis* may cause up to 20% of pertussis-like disease in young

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children, and co-infection with *B. pertussis* is common (4−7). Contemporary options for the detection of *B. pertussis* and *B. parapertussis* infections include immunoserological tests to detect either bacterial antigens in respiratory specimens or pathogen-specific antibodies in serum, culture of nasopharyngeal specimens using specialized media, and nucleic acid amplification tests to detect *B. pertussis* and *B. parapertussis* DNA in nasopharyngeal swab and aspirate specimens (5−7). Although culture affords excellent specificity (nearly 100%), it requires up to 7 days to obtain results, is often labor-intensive, and can have poor sensitivity, especially if specimens were obtained after the initiation of antimicrobial therapy. Also, culture is most successful during the first 1−2 weeks following cough onset in unvaccinated patients that have not received antibiotics (7). Serologic tests for pertussis infection can be helpful, but have limited utility early after infection, and no commercial kits have been approved by the U.S. Food and Drug Administration (FDA) for diagnostic use (8). Molecular assays for detection of *B. pertussis* and *B. parapertussis* detection are much faster than culture, offer higher sensitivity, are continuously improving in test turnaround time and protocol simplification, and have received FDA clearance. The CDC Best Practices guidelines indicate that PCR-based assays are recommended as first-line approaches for diagnosing pertussis in symptomatic patients (9).

The ARIES *Bordetella* Assay (Luminex Corp., Austin, TX) is an automated nucleic acid amplification assay that is designed to rapidly, simultaneously, and differentially identify *B. pertussis* and *B. parapertussis* in nasopharyngeal swab specimens. The assay detects the pertussis toxin (*ptxA*) promoter and IS*1001* of *B. pertussis* and *B. parapertussis*, respectively. The current study evaluated the analytical performance of the ARIES *Bordetella* Assay system by conducting a large-scale multi-site evaluation of the assay using clinical specimens obtained from subjects with suspected and/or known pertussis infection.

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MATERIALS AND METHODS

Clinical study design.

Inclusion Criteria and Study Oversight. Inclusion criteria were subjects: i) with signs and symptoms consistent with *B. pertussis* and/or *B. parapertussis* infection; ii) for whom a *Bordetella* test had been requested; and iii) who provided a nasopharyngeal swab specimen collected in Universal Transport Medium (UTM) with volume ≥1,000 µL. Specimens were excluded if subjects had antibiotic use within 24 h of specimen collection.

Clinical specimens were exempted from informed consent requirements by the Institutional Review Board at all participating sites per FDA advisory *Guidance on Informed Consent for In Vitro Diagnostic Device Studies using Leftover Human Specimens that are not Individually Identifiable, January 2006* (10); results were not used for patient management. Chart review data were anonymized before compilation and analysis. This study was registered on ClinicalTrials.gov (NCT02862262), and conformed to the Declaration of Helsinki and the Health Insurance Portability and Accountability Act.

Specimens. Diagnostic accuracy of the ARIES *Bordetella* Assay was evaluated in 1,053 prospectively collected, de-identified nasopharyngeal swab specimens from subjects presenting with suspected *B. pertussis* or *B. parapertussis* infection at five geographically diverse sites in the United States (New Mexico, Wisconsin, Ohio, Michigan, and Indiana) between July and November, 2015. The Ohio samples were assayed at the New Mexico site. Investigators, technicians performing *Bordetella* testing, and the study sponsor were masked with respect to specimen identification. Swab specimens were collected in UTM, refrigerated at 2−8ºC within 4

h of collection, and specimen aliquots (≥250 µL) were stored at 2−8ºC (and tested within 72 h) or frozen at -70ºC for testing at a later time.

Study Groups. This clinical study comprised three study arms. Arm 1 was the blinded prospective evaluation of nasopharyngeal swabs collected from subjects with symptoms of *B. pertussis* or *B. parapertussis* infection, but without accompanying diagnostic information. We estimated *B. pertussis* to be present in 5% of samples in Arm 1. Because this frequency was not guaranteed, per protocol we generated a second arm, Arm 2, containing additional banked pre-selected known *Bordetella*-positive swab specimens (37 *B. pertussis* and 20 *B. parapertussis*) and an equal number (n=57) of unique *B. pertussis-negative*/*B. parapertussis*-negative clinical specimens from study sites. All Arm 2 samples had been stored frozen and were evaluated in random order. Because *B. parapertussis* is particularly rare, and we wished to accurately estimate positive and negative percent agreement (PPA and NPA, respectively) between the ARIES and reference assays, Arm 3 contained 50 contrived specimens that were spiked with *B. parapertussis* strains at clinically-relevant titers, and tested in random order among 50 negative-control specimens.

ARIES *Bordetella* Assay operation. All study sites used the ARIES system with *Bordetella* Assay Cassettes (Luminex) and ARIES *Bordetella* Assay Protocol Source File-IUO v.5. Analysis software was SyncT ARIES UDP Desktop Software, Version 1.1 Build 165 (Luminex). Four specimen aliquots (≥250 µL) were stored at 2−8ºC, and tested within 72 h of collection or frozen (-65°C to -95°C), per manufacturer instructions (11). External positive-controls included pooled *B. pertussis* A639 and *B. parapertussis* A747 cultures diluted in Natural Negative

Nasopharyngeal Matrix (NPM); negative-controls were vehicle-only. The assay cassettes contain extractable Sample Processing Controls to assess PCR extraction and amplification fidelity. Specimens testing negative for *Bordetella* were required to have a positive result for these internal controls (cycles, Ct; and melting temperature, T_m) for the negative result to be validated.

Comparator assays. One frozen aliquot was shipped on dry ice to Luminex Molecular Diagnostics (Toronto, ON) for comparator testing, with two frozen aliquots remaining on site as reserves. All ARIES assay results, whether positive or negative, were confirmed by two real-time PCR assays per organism (total = 4 PCR assays), with results validated by conventional (endpoint) PCR amplification followed by bidirectional sequencing of the amplicons, in accordance with FDA guidance. The reference real-time PCR assays for *B. pertussis* and *B. parapertussis* targeted unique regions of the *ptxA* gene promoter and *IS1001* insertion sequence, respectively, which differed from the regions targeted by the ARIES assay. Specimens were considered positive for *B. pertussis* or *B. parapertussis* if one or both comparator PCR assays was positive (Ct values ≤40) and confirmed by bidirectional sequencing. All samples from all three arms were evaluated using the ARIES system, as well as by the comparator assays.

Arm 1 samples had previously been tested by each site using their in-house standard-of-care (SOC) assay. Each site used their own laboratory-developed real-time PCR assay targeting IS*481* and IS*1001* for *B. pertussis* and *B. parapertussis*, respectively, with initial findings remaining undisclosed until study terminus. Two study sites performed a laboratory-developed test (LDT) using Luminex MultiCode Analyte Specific Reagents, one site used a TaqMan-based (Thermo-Fisher; Foster City, CA) LDT, and one site used DiaSorin reagents (DiaSorin; Cypress, CA) for their LDT.

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154 **Analytical performance overview**. The AIRES *Bordetella* Assay was evaluated for limit-of-
155 detection (LoD), intra-lab/inter-operator and site-to-site reproducibility, detectability of multiple
156 *B. pertussis* and *B. parapertussis* strains, potential interfering substances and microorganisms,
157 run-to-run carryover, swab and media interference effects, and sample stability during storage,
158 with outcomes detailed in the Luminex AIRES Bordetella Assay 510(k) FDA Decision Summary
159 (12). Reference bacterial strains *B. pertussis* A639 and *B. parapertussis* A747 from
160 (ZeptoMetrix, Buffalo, NY) were used in all assays except where noted. Dilution medium and
161 vehicle-only negative-controls were NPM, derived from nasopharyngeal swab extracts pooled
162 from 242 asymptomatic individuals (BioIVT, Westbury, NY) or UTM, unless otherwise
163 indicated. Data points are the average of triplicate determinations for a given condition, unless
164 otherwise noted.

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166 **Limit of detection.** Six 20-fold serial dilutions of two *B. pertussis* strains (A639 and ATCC
167 BAA-589) and two *B. parapertussis* strains (A747 and ATCC BAA-587 [ATCC, Manassas,
168 VA]) in NPM were assayed to estimate the LoD; this concentration was subsequently tested in
169 20 replicates, and the analytical LoD was defined as the concentration in CFUs that had ≥95%
170 positive detection rate.

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172 **Cross reactivity.** Cross-reactivity of the ARIES *Bordetella* Assay was evaluated in 18 strains of
173 *B. pertussis* and *B. parapertussis* in samples created with 3×LoD, 10×LoD, and 100×LoD of
174 these organisms (Supplementary Table S1). We also evaluated erroneous detection of high-
175 concentration suspensions (in NPM) of 71 non-*B. pertussis/B. parapertussis* potential cross-

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reacting organisms (CROs), many of which cause infections with symptoms resembling those of *Bordetella* infection (12). This included six additional *Bordetella* species, including 4 strains each of *B. bronchiseptica* and *B. holmesii*, at concentrations ranging between 1.0×10^6 CFU·mL^{-1} to 1.9×10^6 CFU·mL^{-1}.

Microbial interference was evaluated by adding the same potential CROs at the same concentrations as in cross-reactivity testing to *B. pertussis* and *B. parapertussis* suspensions containing 3×LoD of either target *Bordetella* pathogen. Cross-target interference in simulated co-infection scenarios was assessed by assaying suspensions containing 100×LoD of both target organisms (high/high) or 3×LoD of both (low/low). For high/low co-infection testing, high concentrations ($\geq10^6$ CFU·mL^{-1}) of *B. pertussis* or *B. parapertussis* were spiked into 3×LoD suspensions of the other *Bordetella* species.

Specimen stability. We evaluated positive detection of 3×LoD *B. pertussis* and *B. parapertussis* samples suspended in NPM that were stored at 20 - 25°C for 0, 2, 4, 8 and 10 h, or at 4 - 8°C for 0, 1, 3, 7, and 10 days, or after storage at -70°C for up to 6 months (n=3 samples per timepoint under each condition). In a separate experiment, suspensions of *B. pertussis* or *B. parapertussis* (3×, 10×, and 100×LoD) were assayed immediately or after freezing at -70°C for 2 days (15–30 replicates per condition/time). In total, 214 specimens were evaluated by the clinical testing sites for stability under freezing conditions (114 from the Arm 2 prospectively collected samples and 100 from the Arm 3 pre-selected retrospective samples).

Statistical analysis. To ensure sufficient statistical power, we calculated the required number of prospectively collected specimens to achieve ≥95% sensitivity with a lower bound of the

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two-sided 95% confidence interval (CI) >85%. Based on an estimated *B. pertussis* prevalence of approximately 5% in the intended population, we calculated that 1,000 specimens were needed to obtain ≥50 positive specimens. Data are presented as n (%) or value ± 95%CIs, as indicated. Positive and negative percent agreement (PPA and NPA, respectively) of the ARIES assay with comparator assays were derived from 2×2 contingency tables, with minimum desired ≥95% PPA with lower bound of two-sided 95%CI >85%. Statistical software included SyncT UDP desktop software v.1.1, build 165 (Luminex), SAS v.9.2 (SAS Corp, Cary, NC), and Prism v.5.0 (GraphPad Software, La Jolla, CA).

RESULTS

Clinical trial subject and specimen accounting. A total of 1,053 unique nasopharyngeal swab specimens from 1,053 subjects presenting with pertussis-like disease were collected at five study sites during July – November 2015. Individual sites provided between 25–427 (2–41%) of total prospective Arm 1 samples. One specimen was excluded from performance calculations due to refrigerator storage >72 h, leaving 1,052 evaluable specimens. Most subjects (74%) were <18-years-old, 56% were female, and nearly 74% visited outpatient clinics (Table 1). ARIES testing was performed on 63.4% of samples (n=667) within 72 h of storage at 4–8°C; the remaining 36.6% (n=385) were stored frozen at -70°C for up to 12 days prior to assay.

Comparison to SOC results. After completing the clinical performance assessment of the study, we compared the results of the ARIES assay to the result initially obtained by each site using their SOC test method. Of the 1,052 evaluated specimens in Arm 1, 23 samples were negative for *B. pertussis* by ARIES and by the ARIES-positive comparator sequencing assay, but had

initially tested *B. pertussis*-positive by the study sites' in-house SOC assay for *IS481*. These samples were further tested by Luminex using a validated high-sensitivity endpoint nested-PCR assay, followed with bidirectional sequencing for *B. pertussis* using primers targeting regions distinct from those used in the ARIES assay and in the clinical trial comparator sequencing assay, to reassess *B. pertussis*. In addition, validated single-plex real-time PCR assays and bidirectional sequencing were performed for *B. holmesii* and *B. bronchiseptica*. Of the 23 discordant specimens, this analysis identified 5 *B. pertussis*-positive and 3 *B. holmesii*-positive specimens (Table 3).

Clinical performance. Of 1,052 unique specimens in prospective Arm 1, 1,043 (99.1%) yielded valid results on the first run. The remaining 9/1,052 (0.9%) initially produced invalid results due to run failure or instrument error, but generated valid results (positive or negative) upon re-testing. The ARIES assay produced 30 positive *B. pertussis* results and 1,011 negative results (Table 2). Compared to the PCR/sequencing comparator controls, the PPA for *B. pertussis* detection was 93.8% and the NPA was 98.9%. For *B. parapertussis* detection, the ARIES assay identified two positives in this population, yielding a PPA of 100% and an NPA of 98.9%. Because the observed prevalences of *B. pertussis* and *B. parapertussis* in the prospective study were lower than expected (3.0%, 32/1,052; and 0.2%, 2/1,052, respectively), the initial study design (Arm 1, prospective) did not achieve the desired minimum criteria of ≥95% PPA with a lower bound of the two-sided 95% CI >85%. Per protocol, we supplemented this sample set with pre-selected specimens that tested positive for *B. pertussis* and *B. parapertussis* by comparator assays (Arm 2; pre-selected). In addition, we tested *B. parapertussis* suspensions at clinically relevant titers (Arm 3, contrived).

Percent agreement to a reference method in part depends on the frequency of the studied pathogen. To increase *Bordetella* frequency, a set of 57 pre-selected nasopharyngeal swabs (37 for *B. pertussis* and 20 for *B. parapertussis*) were obtained from study sites that had been previously characterized as positive for these pathogens. These Arm 2 samples underwent ARIES *Bordetella* testing at 3 study sites, with comparator real-time PCR and bidirectional sequencing performed at Luminex. An equal number of clinical samples negative for *B. pertussis* and *B. parapertussis* (n=57) were included as controls. All specimens were anonymized and their order of analysis was randomized. The PPA for *B. pertussis* in the 37 pre-selected samples was 100%, with a lower bound of the 95% CI: 90.5%; the NPA was also 100%, with lower 95% CI bound: 95.3% (Table 2). In the 20 pre-selected *B. parapertussis* samples, the PPA was 100%, although the lower 95% CI bound was 83.2%; the NPA was 99% with the lower 95% CI bound: 98.9%. In 50 contrived *B. parapertussis* samples in Arm 3, both PPA and NPA were 100%, with the lower CI bound satisfying the desired criterion (Table 2). After pooling results from all study arms, the PPA and NPA for both *B. pertussis* and *B. parapertussis* satisfied all pre-defined acceptability criteria.

LoD. The LoD was defined as the lowest concentration of *Bordetella* that was detectable in ≥ 95% of 20 replicates. In *B. pertussis* strains A639 and BAA-589, LoDs were 1,640 CFU·mL^{-1} and 1,800 CFU·mL^{-1}; in *B. parapertussis* strains A747 and BAA-587, LoDs were 172 CFU·mL^{-1} and 213 CFU·mL^{-1}, respectively. Thus, 1,800 CFU·mL^{-1} and 213 CFU·mL^{-1} were considered to be the LoDs for *B. pertussis* and *B. parapertussis*, respectively.

Cross-reactivity. Of the 71 unique potential CROs tested alone, 66 were non-reactive with the ARIES *Bordetella* Assay at high titers, including 4 strains each of *B. bronchiseptica* and *B. holmesii* (12). All of the 5 CROs (*Fusobacterium necrophorum* and *Proteus vulgaris* for *B. pertussis*, and human coronavirus OC43, influenza B virus, and *Moraxella catarrhalis* for *B. parapertussis*) generated a false-positive in 1/3 replicates each, all with late Cts (38−40 cycles); no cross-reactivity was observed when these CROs were assayed in 3 additional replicates.

Cross-reactivity of the ARIES *Bordetella* Assay was also evaluated in 18 strains of *B. pertussis* and *B. parapertussis* (Supplementary Table S1). Nine of 11 (81.8%) *B. pertussis* strains were detected at 100% positivity at 3×LoD while two *B. pertussis* strains, ATCC 8478 and ATCC 9797, were not detected at up to 100×LoD. All seven *B. parapertussis* strains were detected at 100% positivity when tested at 3×LoD.

In interference assays with *Bordetella* suspensions spiked with CROs, *B. pertussis* was correctly detected in 3/3 (100%) replicates in the presence of 66 CROs. Five CROs in total, *B. bronchiseptica* (strains 1 and 2), *Bordetella petrii*, *Klebsiella aerogenes*, and *Klebsiella pneumoniae* required testing of 3 additional replicates, per protocol. For these 5 CROs, *B. pertussis* was detected in 5/6 (83.3%) replicates. *B. parapertussis* was correctly detected in 3/3 (100%) replicates when tested in presence of all 71 CROs.

Microbial interference was also evaluated in simulated *B. pertussis*/*B. parapertussis* co-infection settings with variable ratios of *B. pertussis* to *B. parapertussis* (*e.g.*, high [100×LoD] -low [3×LoD], low-high, high-high, and low-low). All replicates in all combinations yielded expected positivity for *B. pertussis* and *B. parapertussis* (12). The overall invalid rate was 0.9% (7/744) during the microbial interference and cross-reactivity study; all invalids were re-run and gave the expected result.

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Specimen stability. One-hundred percent of both *B. pertussis*- and *B. parapertussis*-positive (3×LoD) and NPM-only negative controls were accurately distinguished after storage at 20–25°C for at least 10 hours, 4–8°C for at least 10 days, and -70°C for up to 6 months (n=3 samples per timepoint under each condition; not shown). No differences were seen in detection of identical suspensions of *B. pertussis* or *B. parapertussis* (3×, 10×, and 100×LoD) that were assayed immediately after production or after freezing at -70°C for 2 days (12).

DISCUSSION

This study characterized the performance of the ARIES *Bordetella* Assay for identifying and differentiating *B. pertussis* and *B. parapertussis* in nasopharyngeal swabs from patients with suspected or known pertussis. Whereas microbiological culture of nasopharyngeal swab or aspirate samples is the most specific means of detecting and delineating *B. pertussis* and *B. parapertussis*, these pathogens can be fastidious to grow *in vitro* and frequently require 5–7 days to acquire results (5–7, 13). This time lag delays implementation of appropriate antimicrobial therapy and infection control measures, often when the infected individual is most contagious (2, 3). While the World Health Organization (WHO) surveillance guidelines still include paired serology as a *B. pertussis* diagnostic measure, this requires that sequential sampling and testing occur at least 1 week apart (14), conferring the same delay in obtaining results that occurs with culture. The CDC does not recognize a role for serological testing in its pertussis surveillance guidelines (7) and essentially all current *Bordetella* surveillance guidelines concur on the evident utility of nucleic acid amplification testing as a first-line approach for diagnosing *Bordetella*

infection (9, 14, 15). The ARIES *Bordetella* Assay accurately identifies two primary *Bordetella* pathogens in less than 2 hours.

Molecular diagnostic tests for identifying *Bordetella* species in nasopharyngeal swabs and aspirates are more sensitive than serological testing, provide faster results than culture, and are applicable to specimens collected up to 3 weeks after the onset of cough. Nucleic acid amplification assays can potentially match culture's high specificity and ability to simultaneously identify the presence of multiple *Bordetella* species (9, 16–18). The *B. parapertussis* infection rate is increasing, thought in part to be due to a competitive advantage conferred by vaccination against *BP* (4, 6, 19). The overlapping symptomatology caused by *B. pertussis* and *B. parapertussis* increases the importance of being able to confidently identify and differentiate these two most common human-pathogenic *Bordetella* species.

The ARIES *Bordetella* Assay consistently detected the presence of low titers of 9 *B. pertussis* and 7 *B. parapertussis* strains. Two *B. pertussis* strains that went undetected contained similar nucleotide mismatches in the ARIES primer binding regions (19), which presumably hindered assay detection of these strains. A search of the National Center for Biotechnology Information database revealed a very low (2.9%) prevalence of two similarly mismatched *B. pertussis* strains, both collected over a decade ago, and only one of which is known to have a human origin. This suggests a current paucity of similar mismatched *B. pertussis* strains circulating in the human population. Coinfection with *B. pertussis* and *B. parapertussis* occurs and might affect both treatment and outcome (20, 21). The ARIES assay was able to detect both of these organisms at low titers in the presence of extremely high concentrations of the sister pathogen, thereby demonstrating utility for accurately characterizing the most likely *Bordetella* species coinfection scenarios.

The ARIES *Bordetella* Assay has a low likelihood of falsely detecting a potential CRO as *B. pertussis* or *B. parapertussis*, or of a CRO interfering with intended *B. pertussis*/*B. parapertussis* detection, even near the *Bordetella* species' LoDs. The single-copy *ptxA* gene is present only in *B. pertussis*, which confers increased specificity to the ARIES assay in accurately diagnosing true pertussis compared to nucleic acid assays that target IS*481*, a multi-copy gene that is also present in the confounding pathogens *B. holmesii* and some strains of *B. bronchiseptica* (22−25). Of other currently FDA-cleared *B. pertussis* diagnostic assays, one system (FilmArray RP and RP2; BioFire Diagnostics) targets the *ptxA* gene, while another (Illumigene; Meridian Bioscience) targets the IS*481* gene and is known to additionally detect *B. holmesii* and *B. bronchiseptica* (26, 27).

The ARIES assay identifies *B. parapertussis* by detecting IS*1001*, present at approximately 20 copies per genome (17). Although IS*1001* has been reported in occasional strains of *B. bronchiseptica* (4/73 human isolates), this low expression level coupled with the very rare involvement of this organism in suspected pertussis outbreaks makes it unlikely to be a meaningful impediment to ARIES diagnostic utility (23, 28). The BioFire RP2 also evaluates IS*1001*; the Illumigene does not assess *B. parapertussis*. *B. bronchiseptica* was not detected in any sample in the current study.

Of 71 potential CROs assayed, 66 (93%) were not detected by ARIES. Two organisms produced a single false-positive for *B. pertussis* that was not replicated in triplicate re-testing; similarly, three CROs generated a single false-positive for *B. parapertussis* that was not repeatable. Human specimens and contrived samples remained stable during common storage temperatures and durations. Together these observations demonstrate the ARIES assay's

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reliability in selectively identifying low titers of the two most common *Bordetella* pathogens in diverse testing scenarios.

We reconciled ARIES findings by comparison to results from two unique real-time PCR reference assays for each pathogen (four assays total), supplemented with bidirectional sequencing of PCR amplicons when either one or both of the two PCR assays (per organism) was positive. In the prospective arm, 99.1% of specimens (1,043/1,052) yielded valid results on the first attempt, and remaining nine samples provided a definitive conclusion when retested. Because our population contained lower than the estimated *B. pertussis* incidence of 5%, we supplemented our sample set with known *B. pertussis*-positive (n=37) or *B. parapertussis*-positive (n=20) clinical samples to increase pathogen frequency; all were accurately assessed by the ARIES *Bordetella* Assay. Because *B. parapertussis*-positive samples were expectedly lower in number than *B. pertussis*-positive samples, we also included 50 contrived *B. parapertussis* specimens to increase the accuracy of our percent agreement calculations, along with appropriate vehicle-only controls, all of which were also accurately identified. The ARIES' cumulative PPA and NPA exceeded the minimum acceptable performance criteria by a substantial margin. The respective PPAs for *B. pertussis* and *B. parapertussis* were 97.1% and 100%, with lower 95% CI bounds above the minimum 85% specification for both organisms. The respective NPAs for *B. pertussis* and *B. parapertussis* were 99.0% and 99.7%, respectively, again with lower 95% CI bounds above 85% for both species. Thus, the diagnostic accuracy of the ARIES *Bordetella* Assay is acceptable for effectively detecting *BP* and *BPP* in nasopharyngeal swabs from patients suspected of having respiratory tract infection attributable to either pathogen.

Initial SOC testing performed by each study site produced 23 cases of putative *B. pertussis* infection in Arm 1 (23/1,502=1.5%) that were not detected as *B. pertussis*-positive by

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ARIES or by the bidirectional sequencing comparator. All study sites used PCR assays that targeted IS*481*, an insertion sequence present in high copy number in *B. pertussis* (≥50 copies/genome) but that is also present in *B. holmesii* at a lower copy number (*e.g.*, 8–10 copies/genome) (22, 25). Highly sensitive follow-up testing of the 23 discordant samples identified 5 additional *B. pertussis*-positive specimens, 3 *B. holmesii*-positive specimens, and 15 confirmed *Bordetella*-negative samples. This reflects the utility of IS*481* assays as a sensitive screening tool for *B. pertussis*, but also highlights its limited diagnostic specificity that can potentially misidentify other less insidious *Bordetella* spp. as true pertussis. For example, *B. holmesii* was present in up to 20% of suspected pertussis cases during a 2009–2011 outbreak in France (29). Whereas only approximately 5% of *B. bronchiseptica* strains reportedly express IS*481*, a commonly used primer set used for PCR detection of IS*481* erroneously produced diagnostic amplicons of the predicted size for this gene in 78% of 149 tested *B. bronchiseptica* isolates, including in 22/24 that were of human origin (24). The IS*481* assay may also produce false-positive *B. pertussis* findings by detecting high-copy IS*481* DNA on lightly contaminated laboratory surfaces (30), or even from aerosolized pertussis vaccines in the clinic setting in the absence of viable organisms being present in samples (31). Interestingly, 12/15 (80%) of the study site SOC IS*481*-positive samples that ultimately tested negative for *B. pertussis* had initial IS*481* Ct values >35.0 (Table 3), suggesting that it detected a low total starting target sequence copy number. Together, these limitations of IS*481*-based assays may be responsible for identifying pertussis pseudo-outbreaks that do not truly constitute public health emergencies (32), and may constitute more of a public health concern than the low false-negative rate using the *ptxA*-based ARIES assay.

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Study strengths include the multicenter prospective design, a large initial clinical sample size and the availability of supplemental known-positive *BP* and *BPP* clinical samples necessary to increase the accuracy of PPA and NPA determinations in low-frequency illnesses, particularly with *BPP*, and the availability of three robust reference techniques for comparison with ARIES findings. Study limitations include the inability of the qualitative ARIES assay to discern symptomatic, subclinical, and resolving *Bordetella* infection, although the likelihood of misidentifying an active infection is negated by judicious evaluation of clinical symptoms (5–7). The low natural prevalence of *B. pertussis* and *B. parapertussis* in the prospectively collected samples required inclusion of additional specimens with known *Bordetella* PCR outcomes or the use of contrived samples. Because the ARIES assay targets genetic sequences specific to *B. pertussis* and *B. parapertussis*, it is unable to identify the two other *Bordetella* species that can cause pertussis-like symptoms in humans, *i.e.*, *B. bronchiseptica* and *B. holmesii*, although infection with these organisms is comparatively rare (4–6).

The ARIES system can simultaneously evaluate up to 12 samples with minimal hands-on time required during its 2-hour runtime. The self-contained assay cartridges contain all reaction components and an internal sample processing control to monitor PCR fidelity. This study demonstrated that the ARIES *Bordetella* diagnostic assay reproducibly and accurately detects and discerns the two most clinically relevant *Bordetella* species that cause pertussis-like disease.

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Table 1 Demographics of the clinical study population in prospective Arm 1.

Gender	Site 1	Site 2	Site 3	Site 4	Site 5	All Sites
Male	56 (40.9%)	180 (42.3%)	56 (45.2%)	11 (44.0%)	164 (48.2%)	467 (44.4%)
Female	81 (59.1%)	246 (57.7%)	68 (54.8%)	14 (56.0%)	176 (51.8%)	585 (55.6%)
Age, yrs						
<18	76 (55.5%)	263 (61.7%)	92 (74.2%)	22 (88.0%)	326 (95.9%)	779 (74.0%)
≥18	61 (44.5%)	163 (38.3%)	32 (25.8%)	3 (12.0%)	14 (4.1%)	273 (26.0%)
Subjects by Site	137	424	124	25	339	1052

Table 2 ARIES *Bordetella* Assay PPA and NPA for *B. pertussis* and *B. parapertussis* detection versus comparator real-time PCR assays and bidirectional sequencing.

Bp	**PPA**		**95% CI**	**NPA**		**95% CI**
Prospective (Arm 1)	30/32[a]	93.8%	79.2%–99.2%	1,009/1020	98.9%	98.1%–99.5%
Pre-selected (Arm 2)	37/37	100%	90.5%–100%	77/77	100%	95.3%–100%
Total	67/69	97.1%	89.9%–99.6%	1,086/1097	99.0%	98.2%–99.5%
Bpp	**PPA**		**95% CI**	**NPA**		**95% CI**
Prospective (Arm 1)	2/2	100%	15.8%–100%	1,048/1050	99.8%	99.3%–100%
Pre-selected (Arm 2)	20/20	100%	83.2%–100%	93/94[b]	98.9%	94.2%–100%
Contrived (Arm 3)	50/50	100%	92.9%–100%	50/50	100%	92.9%–100%
Total	72/72	100%	95.0%–100%	1,191/1194	99.7%	99.3%–99.9%

[a]Two prospective specimens generated false-negative results by ARIES *Bordetella* assay when compared to the composite comparator method. One of these two specimens gave a positive result in only one of two comparator real-time PCR assays. The other specimen generated Ct values between 37.1 and 38.8 by comparator real-time PCR assays; in this specimen, the ARIES *Bordetella* assay detected low levels of DNA (Ct=40.1), which was close to the assay cut-off.

[b]One pre-selected specimen generated a false-positive *BPP* result by ARIES *Bordetella* assay that was not confirmed by comparator real-time PCR and bi-directional sequencing.

Bp, *B. pertussis*; *Bpp*, *B. parapertussis*; NPA, PPA, positive percent agreement; NPA, negative percent agreement; CI, confidence interval.

Table 3 Reconciliation results of 23 discordant samples that were negative by ARIES for *B. pertussis*, but positive by each study site's standard-of-care IS*481* PCR assays.

Study Site	Sample	Sequencing Result	Site *IS481* Assay Ct
Site A	02-003	POS *Bordetella pertussis**	
	02-006	NEG	37.0
	02-007	POS *Bordetella holmesii* †	
	02-037	POS *Bordetella pertussis**	
	02-059	NEG	37.3
	02-076	POS *Bordetella pertussis**	
	02-103	POS *Bordetella pertussis**	
	02-119	NEG	36.9
	02-165	NEG	36.5
	02-407	NEG	38.1
	02-501	NEG	38.6
Site B	05-005	NEG	40.1
	05-013	NEG	38.5
	05-024	NEG	39.2
Site C	06-031	NEG	36.7
	06-076	NEG	36.0
	06-125	POS *Bordetella holmesii*	
	06-156	NEG	32.8
	06-172	NEG	31.2
	06-174	POS *Bordetella pertussis*	
	06-179	POS *Bordetella holmesii* †	
	06-314	NEG	33.5
	06-332	NEG	36.9

* Detected in the forward read only

† Detected in the reverse read only